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13. ABSTRACT (Maximum 200 Words) Exposure to specific neurotoxins can cause Parkinson's disease by killing dopaminergic neurons. These neurotoxins can cause neuronal loss by inducing apoptosis in which cell death results from sequential signalling events leading from the inducer of apoptosis to the ultimate execution of the cellular changes characteristic of this process. Evidence from our laboratory and others suggest that activation of dopamine receptors can oppose the induction of apoptosis in dopamine neurons. By integrating molecular biological, pharmacological, cell biological and physiological approaches, the points of intersection of the neurotoxin-activated pathways mediating cell death and the dopamine receptor-mediated pathways that promote survival will be investigated. The locus at which dopamine receptor activation interferes with the concatenated events mediating neurotoxin-initiated apoptosis will be determined. The sequence of events leading from activation of a specific dopamine receptor to modulation of apoptosis will be investigated. This work will identify specific molecular targets for drugs that oppose neurotoxin-mediated cell loss. The results of these studies can facilitate the development of effective, rational interventions for Parkinson's disease resulting from neurotoxin exposure. Accomplishments include establishing real-time PCR and developing and testing microarray technology for these paradigms.				
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Introduction:

The purpose of this research is to investigate the sequence of events leading from activation of specific dopamine receptors to modulation of apoptosis. Neurotoxin-induced apoptosis represents a signaling cascade leading from initial insult to the irrevocable commitment to execute the cell-death program. Data from our group and others have shown that DA receptor activation can decrease the probability of a cell entering apoptosis. The investigation of the underlying mechanisms for this effect may lead to improvements in the ability to modulate this process and protect neurons against neurotoxin-mediated cell death. In neurotoxin-mediated cell death, apoptosis represents a stochastic two-state outcome resulting from an initial stimulus. In other words, whatever the level of neurotoxin exposure, the result is limited to two states--an individual cell either survives or executes the cell death program. This binary decision making occurs as a result of the activity in a network of signaling mediators. Thus in order to begin to understand neurotoxin-induced apoptosis and its modulation by dopamine agonists in a deterministic fashion, we need to develop ways to monitor the overall perturbation of the cell's signaling network. A major focus of this grant is to develop the capability of monitoring the overall physiological state of the cell by following changes in gene transcription. This approach will represent a fundamental advance in the field and has been the primary focus for the first year of work on the project.

Body:

Following in italics is the statement of work from June 1999. Work during the first year focused on objective IB and objective 2A. We undertook monitoring of gene changes in primary neuronal cultures in response to dopamine agonists and in PC12 cells in response to trophic factors. We developed microarray technology and explored its limitations and developed quantitative PCR assays to allow precise monitoring of gene changes in response to graded stimuli. One review article and one abstract based in part on the work performed under support from this grant have been published (see appendix). Details of our progress are presented below.

Objective 1: Determine the locus at which dopamine receptor activation interferes with the concatenated events mediating neurotoxin-initiated apoptosis

A. The neuroprotective effects of DA agonists on DA neuron survival in primary culture will be determined.

- B. The effects of DA agonists on gene program changes in primary culture will be determined*
- C. The neuroprotective effects of dopamine agonists in PC12 cells will be characterized pharmacologically.*
- D. The effects of DA agonists on mitochondrial function in PC12 cells in the presence and absence of apoptosis-initiators will be determined.*
- E. The effects of DA agonists on other changes leading to apoptosis will be determined, including: Glyceraldehyde-3-phosphate dehydrogenase translocation and NF- κ B activation*

Objective 2: Determine the sequence of events leading from activation of a specific dopamine receptor to modulation of apoptosis.

- A. The specific DA receptor mediating neuroprotection will be investigated by antisense experiments in PC12 cell and primary culture.*
- B. The second messenger systems involved in DA agonist mediated neuroprotection in PC12 cells will be investigated. These include:*
 - i. heterotrimeric G-proteins*
 - ii. low molecular weight G-proteins*
 - iii. calcium*
 - iv. cAMP*
 - v. tyrosine kinase activation*
 - vi. serine/threonine kinase activation*
 - vii. alterations of actin cytoskeleton*
 - viii. alterations in gene expression pattern*

I. Investigation of overall gene changes in primary cultures in response to dopamine agonist.

Objective: Determine the pattern of gene changes in response to dopamine agonist in primary neuronal cultures using gene microarrays.

Experimental design: These experiments utilized the filter-based microarray provided commercially by Research Genetics. This array has >5000 cDNAs for which the relative level of expression can be determined by comparative hybridization. A series of experiments were performed to examine the different filters available and to evaluate changes of genes over time and at different concentrations of the dopamine agonist pramipexole.

Experimental Results:

1. human gene filter, time course of response.

In this study, 5000 genes were assayed using RNA samples from vehicle and 1 μ M pramipexole (a D₁ agonist), with exposure for 1, 3, 6, 9 and 24 h. The hybridizations were very clean. The dataset generated was very large (>50,000 points). However regression analysis of the expression level of the genes in control and treated samples suggested that there was an unfavorable signal to noise in the assay.

Conclusion: Signal:noise was unfavorable using the human gene filters for rat samples. We then undertook a second experiment using the rat cDNA filter, which had just then become available.

2. rat gene filter, time course and concentration-dependence of response.

Samples were obtained at 0, 1 and 6 h from vehicle and cultures treated with 5 concentrations of pramipexole (1 nM to 10 μ M). Triplicate samples were included for analysis to allow evaluation of internal consistency. The samples were assayed using the Research Genetics rat filter microarrays. The labeling and hybridizations were superb, with the raw data looking very clean. Again a large dataset of measurements were obtained. However regression analysis showed considerable measurement scatter and measurements of independent samples in the same experimental groups were variable.

Conclusion: Despite meticulous and rigorous experimental technique, assay scatter remained significant. There were two possibilities for these results a) the commercial microarray assay system being used was inherently unreliable or b) the heterogeneous nature of the primary culture system and/or the relatively subtle nature of the stimulus (dopamine receptor stimulation) did not lead to a discernable signal over background variation. We decided to investigate these two possibilities by establishing a rapid, reliable and independent assay of gene expression levels and by testing an experimental paradigm with known robust gene changes, NGF exposure to PC12 cells.

3. Establishment of real-time PCR.

Real-time PCR was not available at Mount Sinai. This technique allows the rapid and highly quantitative measurement of specific genes. We

established a collaboration with Dr. Sanjay Tyagi at NYU to establish a real time PCR assay. We selected as our experimental protocol NGF treatment of PC12 cells, as this was reported to cause significant up-regulation of a variety of genes. PC12 cells were treated with NGF or vehicle and RNA extracted at various time points from 30 minutes to 6 h. Real time PCR was established using SYBR green incorporation for Actin, Arc and EGR1.

An example of the results is shown below:

Gene	Fold change (relative to vehicle)	
	30 minutes NGF	90 minutes NGF
Actin	1.5	0.6
Egr	254	613
Arc	2.7	39

Conclusions: Real time PCR will allow rapid and precise quantitation of specific gene products. The NGF treated PC12 samples show expected changes in Egr and Arc gene expression and are suitable samples for testing the microarray system.

4. Validation of microarray system using NGF treated PC12 cells.

The samples generated in experiment #3 were assayed using the Research Genetics rat microarray filters. 10 genes that showed regulation on the filter were selected for analysis by real time PCR. The microarray results for the Egr gene, which is present on the filter, were also determined. The Egr gene, which had been demonstrated in the experiment above to be highly regulated, was seen to have only a small and not-significant 1.3 fold increase in the microarray assay. Ten genes which were carefully selected as being regulated on the microarrays were tested by real-time PCR were not in fact found to show any regulation.

Conclusion: The Research Genetics microarray system does not generate an acceptable signal:noise ratio in experiments designed to determine global gene expression, even in a well-characterized paradigm in which highly significant gene regulation is known to occur.

In order to complete the objectives of the proposal, it is necessary to develop another approach to studying global gene expression. Based on the published experience of the Brown laboratory at Stanford and other institutions, developing the capacity to assay custom printed glass microarrays appears to be the most accurate and cost-effective

approach. In order to have the instrumentation necessary for these studies, I assembled a consortium of departments at Mount Sinai and we have purchased a robotic microarray printer and reader (the Affymetrix/GMS system) and a PE7700 real-time PCR instrument, all of which is presently housed in my laboratory. I have also recruited a fellow from the EMBL microarray facility in Heidelberg who has worked extensively with microarray printing and analysis, who will arrive this fall. I have also developed a school-wide microarray facility, to provide a focal point to share the necessary expertise and experience to develop this laboratory approach. We plan to continue to work on genome-wide profiling using both microarray and informatics approaches to identify candidates and to assay the gene changes using real time PCR. We believe we are well poised to make significant and important progress on this research objective over the next two years.

Objective: Determine the DA receptor expression pattern in PC12 cells.

5. Analysis of DA receptors in PC12 cells.

We performed radioligand binding studies on PC12 cell membranes and D2 receptor transfected control membranes. The binding assay worked well, but the level of receptor expression was below the limits of detection of the binding assay. We are presently developing real-time PCR assays to quantify the level of D2,D3 and D4 receptor in PC12 cells before and after differentiation.

Key Research Accomplishments:

1. Validation of commercial Research Genetics microarray system and determination that the system is inadequate to meet the research objectives.
2. Establishment of quantitative real-time PCR technology.
3. Determination that D2 receptor binding sites are below detection limit in PC12 cells.
4. Establishment of in laboratory capability for real time PCR and glass custom microarray printing and reading.

Reportable Outcomes:

Publications:

Soussis IA, Mytilineou C, Olanow CW, Sealfon SC. Pattern of gene induction by dopamine agonists in rat midbrain cultures studied by microarray analysis. Soc. Neurosci. Abstr. 25:331, 1999.

Sealfon SC and Olanow CW. Dopamine receptors: from structure to behavior. Trends Neurosci. 23:S34-40, 2000.

Conclusions:

1. Signal:noise was unfavorable using the Research Genetics human gene microarray filters for rat samples in dopamine agonist treatment experiments.
2. Signal:noise was unfavorable using the Research Genetics rat gene microarray filters for rat samples in dopamine agonist treatment experiments.
3. Signal:noise was unfavorable using the Research Genetics rat gene microarray filters for PC12 cells studied with NGF induced differentiation.
4. The filter microarray system does not have the measurement accuracy required for the proposed studies.
5. Real time PCR will allow rapid and precise quantitation of specific gene products.
6. Based on published literature (eg. [1, 2], fluorescent-based custom printed microarrays are a better approach to meet the objectives of this proposal.

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2. Iyer, V.R., et al., *The transcriptional program in the response of human fibroblasts to serum*. Science, 1999. **283**(5398): p. 83-7.

Appendices:

Soussis IA, Mytilineou C, Olanow CW, Sealfon SC. Pattern of gene induction by dopamine agonists in rat midbrain cultures studied by microarray analysis. Soc. Neurosci. Abstr. 25:331, 1999.

Sealfon SC and Olanow CW. Dopamine receptors: from structure to behavior. Trends Neurosci. 23:S34-40, 2000.

Dopamine receptors: from structure to behavior

Stuart C. Sealton and C. Warren Olanow

The responses obtained with drugs that act at dopamine receptors depend on the spectrum of receptors stimulated, the pattern of stimulation and the neuronal signal-transduction pathways that are activated. In the absence of drugs that reliably discriminate between the various cloned receptors, elucidating the role of these receptors has largely relied on molecular genetic approaches that include expression of genes for receptors in cell lines and manipulation of this expression in animal models. Connecting molecular events that occur consequent to receptor stimulation with the resulting physiological effects entails bridging a complex network of interactions. This article reviews the current understanding of the molecular, cellular and systemic consequences of activation of the different dopamine receptors.

Trends Neurosci. (2000) 23, S34–S40

THE EVIDENCE that lack of dopamine (DA)-receptor stimulation contributes to Parkinson disease (PD) symptoms, and that receptor activation has a beneficial effect on these symptoms appears incontrovertible. The principal effects of L-dopa occur after its conversion to DA via stimulation of various DA receptors. Direct acting DA-receptor agonists that do not require metabolic conversion and act directly at DA-receptor sites, have similarly been shown to provide benefit in PD. However, the receptor stimulation profile, and the resulting clinical benefits of L-dopa and DA-receptor agonists are not identical. For example, clinical benefits are greater with L-dopa, while DA-receptor agonists are less likely to induce motor complications such as dyskinesia and motor fluctuations. In order to understand better how L-dopa and the various DA-receptor agonists modulate behavioral responses differentially, the results of their actions on specific DA receptors must be understood.

Considerable progress has been made in elucidating the anatomy, neurochemistry, physiology, pharmacology and molecular biology of the dopaminergic system and its receptors^{1–3}. DA receptors are members of a large family of structurally related receptors, the rhodopsin-like G-protein-coupled-receptor (GPCR) superfamily. With the recognition that there are multiple DA receptors, it has become evident that the response to a DA-receptor agonist is likely to depend on its relative activity at these molecularly identified receptors. However, determining the relationship between receptor activation and physiological response is difficult. The responses that ultimately occur after receptor activation result from modulation of a complex network of signaling molecules and neuronal circuits. Resolving these issues is complicated by a lack of drugs that are selective for the cloned receptor subtypes, and by the existence of both immediate and long-lasting effects in response to receptor stimulation. For example, treatment with L-dopa is associated with immediate motor benefits and with late-onset motor complications that presumably reflect drug-induced plastic changes in the response network.

The ultimate response to an agonist is determined by the specific receptors that are selected and by the degree

to which signal-transduction pathways are activated. Moreover, this response pattern is likely to be complex: one can activate more than one receptor, receptors can activate more than one signal-transduction pathway, and different agonists acting at the same receptor can differentially affect these signaling pathways. Thus, the effects of an agonist are determined not merely by the spectrum of receptors activated and the degree of activation, but also by the specific pattern of signal transduction that is elicited.

Dopamine-receptor subtypes

D1 and D2 receptors were initially characterized based on differences in ligand selectivity, and positive (D1) or negative (D2) coupling to adenylate cyclase⁴. Molecular cloning revealed the existence of five (D1–D5) DA-receptor subtypes. The cloned D1 and D5 receptors are pharmacologically D1-like, and the genes for the D2–D4 receptors encode a D2-like family of receptors². The rat D2 receptor was the first dopamine receptor to be cloned, being isolated by its homology to the β_2 -adrenergic receptor⁵. Like all DA receptors cloned to date, it has seven hydrophobic domains that constitute predominantly α -helical membrane-spanning segments. The distribution of D2 receptor mRNA has been studied by *in situ* hybridization. Highest levels are observed in the striatum, nucleus accumbens and olfactory tubercle, as well as in dopaminergic neurons in the midbrain, where they presumably generate autoreceptors. The gene for the D2 receptor also gives rise to two receptor isoforms, through alternative exon splicing, that differ in the presence (D_{2L}) or absence (D_{2S}) of a 29 amino-acid segment in the third cytoplasmic loop². These isoforms show differences in G-protein coupling, regional distribution and sequestration rate^{6–8}. In contrast to cloned D2-like receptors, cloned D1 receptors have a short third cytoplasmic loop. Highest levels of D1 receptor mRNA are found in the striatum, nucleus accumbens and olfactory tubercle.

The D3 receptor has about 75% homology to the D2 receptor in the transmembrane domains, but differs in its pharmacological profile, signal-transduction coupling

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and distribution⁹. D3-receptor mRNA levels are lower in the striatum and higher in nucleus accumbens than D2-receptor mRNA. Alternative transcripts of the D3 receptor have been identified, but only mouse alternative transcripts encode functional receptor isoforms.

The sequence of the D4 receptor has approximately 50% homology to D2 receptors in transmembrane domains¹⁰. Levels of mRNA are highest in frontal cortex, amygdala, olfactory bulb and hypothalamus. The human D4 receptor is highly polymorphic, with a 16 amino-acid-repeat segment (two to ten times) in the third cytoplasmic loop domain. A specific repeat number has been correlated with novelty-seeking personality traits¹¹, and increased delusions in psychotic individuals are associated with a high number of repeat segments¹². In rodents, D5 receptor mRNA is primarily located in the olfactory tubercle, hippocampus and mammillary nucleus, and not in the striatum.

D1 and D2 receptors in the striatum are primarily found on medium spiny neurons where they serve to modulate glutamate-mediated activity¹³. D1-receptor-bearing neurons contain substance P and dynorphin, and give rise to neurons comprising the direct striato-pallidal pathway. D2-receptor-bearing neurons contain enkephalin and give rise to neurons that influence the pallidum by way of the indirect pathway. Recent observations suggest that this model might be overly simplistic. There is evidence for extensive collateralization of striatofugal axons¹⁴, the presence of D2 receptors on striatal interneurons that is increased after a lesion of dopaminergic neurons¹⁵, and co-localization of D1 and D2 receptors on striatal medium spiny neurons¹⁶. Furthermore, there is evidence of projections that provide anatomical connections between subtypes of striatal neurons, and these are markedly increased under conditions of dopaminergic denervation or therapy with DA-like agents^{17,18}. These observations are not accounted for in the present model.

Molecular mechanisms of action

The rhodopsin-like GPCRs, which include DA receptors, comprise several hundred homologous proteins that transduce an extracellular signal into intracellular G-protein activation. Members of this hepta-helical protein family are identified by the presence of a series of conserved amino-acid motifs within their transmembrane segments¹⁹. Activation of GPCRs leads to altered receptor conformation. The activated receptor induces GDP-GTP exchange in the α subunit of a heterotrimeric G protein, which causes dissociation of the α and $\beta\gamma$ G-protein subunits and subsequent modulation of various intracellular effector proteins. The simplest model for receptor activity presupposes that GPCRs exist in either an active or inactive conformational state. Agonists exert their effects by preferentially binding to and stabilizing the active conformation. However, there is no *a priori* reason why there should be only a single active receptor conformation. Proteins are capable of assuming a number of different conformations that are distributed according to an 'energy landscape'²⁰. It is probable that there are several different active receptor conformational states, with some being more stable than others. Agonists stabilize active conformations, and different agonists might stabilize different populations of active conformations. Many receptors are known to activate multiple G proteins, and different G proteins might recognize and interact with different active

conformations of the receptor. As DA-receptor agonists stabilize different active conformations, they might direct the receptor stimulus to different G proteins. This has been termed 'stimulus trafficking'²⁰. Alterations in the normal pattern of tonic and phasic DA-receptor activation²¹, as might occur with dopamine depletion and with stimulation of the denervated receptor by therapy with exogenous DA-like agents, might also alter lead to altered signaling and behavioral patterns.

Evidence for stimulus trafficking arises from the reversal of the relative efficacies of agonists in stimulating different signaling pathways via the same receptor. Reversal of potency or efficacy has been identified with several GPCRs. For example, PACAP-27 is more potent than PACAP-38 in stimulating cAMP production via the PACAP receptor, but less potent in stimulating inositol phosphate accumulation²². The efficacies of the 5-HT_{1A}-receptor agonists rauwolscine and ipsapirone are reversed for activation of G_{o12} compared with G_{q13} (Ref. 23). Differential stimulation of the inositol phosphate and arachidonic-acid second-messenger pathways has also been demonstrated for a series of partial agonists at the human 5-HT_{2A} and 5-HT_{2C} receptors. Although signal trafficking has not yet been described for DA-receptor agonists, the presence of this phenomenon in structurally related receptors suggests that they are likely to have similar properties. Thus, the different effects obtained with DA-receptor agonists result from the conformational states that are stabilized by the agonist complexed with its specific receptor and the pattern of signal transduction responses that are elicited by these conformational states.

Site-directed fluorescent labeling of specific conformational states that occur during receptor activation. Site-directed spin labeling has been used to measure the relative movement of helix 3 and helix 6 side chains of rhodopsin during activation²⁵. The results demonstrate that receptor activation is associated with displacement of the cytoplasmic ends of the two helices and a counterclockwise rotation of helix 6. In support of the idea that movements of these helices contribute to receptor activation, rhodopsin activation is blocked when the movement of helices 3 and 6 is restricted by either disulfide crosslinking²⁵ or engineered Zn²⁺ binding sites²⁶. Similarly, a constitutively active β_2 -adrenergic receptor has been shown to manifest increased accessibility of a helix-6 cysteine for chemical modification. This finding is consistent with counterclockwise rotation of this helix during activation. Site-specific environment-sensitive fluorescent labeling studies also suggest that agonists induce conformational changes of helix 3 and helix 6, consistent with a model of GPCR activation in which helix 6 rotates and displaces from helix 3 (Fig. 1)²⁵.

Although high-resolution data are not yet available on any GPCR, the integration of various experimental and computational approaches provides considerable insight into the function of these receptors. Cryo-electron microscopy studies of rhodopsin have identified the orientation of the transmembrane helix domain bundle of these receptors²⁷. Various approaches have been used to map the binding site of DA receptors, including substituted cysteine-scanning mutagenesis, site-directed mutagenesis and receptor chimeras. The binding pocket of DA receptors, common to all neurotransmitter GPCRs, lies within the transmembrane helix domain (Fig. 2). There is evidence that GPCRs can form homo- and

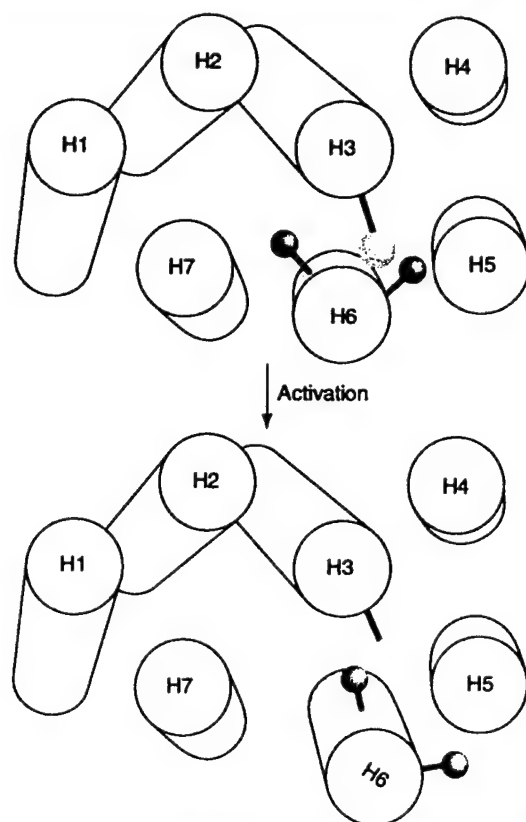


Fig. 1. Movement of a protein complex during activation. A model of the protein complex is shown from the cytoplasmic face. This model is based on electron density maps and spin-labeling studies. Selected helix 3 and 6 side chains are indicated by colored circles. During activation (bottom), helix 6 rotates and displaces with the cytoplasmic side of helix 3. This altered protein conformation leads to interactions with other proteins and activation of intracellular signaling pathways. Adapted from Ref. 25.

heterodimers³⁰, and gel electrophoresis provides evidence of DA-receptor dimers in cell lines and brain samples^{30,31}. GPCRs can also aggregate *in vivo*, and these aggregations can be functionally significant. Using fluorescence resonance energy transfer assays, it can be shown that the somatostatin sst₁ receptor and the D2 receptor form heterodimers that modulate receptor signaling³². For example, signal transduction of a coupling-deficient mutant somatostatin receptor can be restored by co-expression of the gene for the mutant receptor with a D2 receptor, suggesting that the somatostatin receptor can signal through the D2 receptor. Functional interactions between DA receptors and ligand-gated channels have also been reported. The C-terminal domain of the D5 receptor can interact with the second intracellular loop of the GABA_A-receptor $\gamma 2$ subunit, so that each receptor inhibits the function of the other³³.

Molecular genetic approaches to understanding DA-receptor function

DA-receptor agonists and antagonists might not reliably distinguish the various cloned DA receptors. As a result, several laboratories have turned to genetic manipulations to investigate the role of specific DA receptors. Mice with genetically induced lesions that target the D1, D2, D3 and D4 receptors have been

characterized. Antisense sequences for the receptor, using an oligonucleotide or expression vector, can also assess decreased function of a genetically identified receptor. Transgenic receptor overexpression mouse models can be used to assess the effects of gain of function. In this approach, additional copies of the gene of interest are introduced into the pronucleus of a fertilized egg, and the effects of the expression of the transgene are studied. To date, an overexpression transgenic model has only been developed for the gene for the D1 receptor³⁴. There are several caveats that must be considered when evaluating genetic approaches to the study of DA-receptor function. In gene lesion and transgenic approaches, animals develop with a genetic abnormality. Accordingly, the phenotype might be influenced by developmental adaptations or perturbation as well as the background strain in the genetic alteration is studied. These have been proposed to influence the phenotype of D2-receptor deficient mice³⁵. The approach to introduce the genetic lesion can also lead to a different phenotype. In some models, the lesion is completely eliminated, whereas in others, a truncated protein can be produced that does not bind ligands but might retain other nonreceptor functions.

In transgenic mice, the expressed receptor incorporates into a lipid bilayer in order to observe the possibility that receptor activation of another gene and rate of incorporation contributes to the phenotype. It is necessary to study multiple, independently generated lines. In addition, the location and level of expression of the receptor gene is determined by the promoter sequence used for targeting and the site of transgene incorporation. The lack of promoters that predictably target DA receptors to the desired regions has limited this approach. Hybrid arrest approaches attempt to ablate the receptor through the introduction of antisense oligonucleotides or vectors. This approach allows the animal to develop normally, but the reduction in receptor level is incomplete. Furthermore, this approach is restricted by physical limitations in delivery of antisense into target cells. Although these limitations need to be borne in mind, genetic approaches have provided important insights into the role of DA receptors.

Lesions in the gene for the D1 receptor have been reported to induce deficits in movement initiation and reaction to external stimuli in one model^{36,37}, but not in another³⁸. Mice with a genetically lesioned D1 receptor have altered sensitization to amphetamine and do not show the progressive increase in motor responses seen in control animals³⁹. These results suggest that D1 is the predominant receptor involved in sensitization to amphetamine. Deficits in spatial learning and reduced locomotor responses to repeat cocaine injections have also been reported in this model^{40,41}.

Accompanying these behavioral changes, D1-receptor null mice exhibit a reduction in substance P and enkephalin synthesis, increased immunostaining for the GluR1 glutamate-receptor subunit, and decreased staining for the NR1 subunit of the NMDA receptor⁴². Induction of expression of *Fos* and *Junb*, and regulation of dynorphin by cocaine and amphetamine are absent. Interestingly, neurophysiological responses and cellular morphology in the neostriatum are normal in this mouse. However, dopamine does not potentiate NMDA-receptor-mediated electrophysiological responses

In neostriatal slices in D1-receptor-deficient mice¹³. In addition, dopamine-mediated inhibition of glutamate-induced firing is lost in nucleus accumbens neurons. The effects of D2-receptor agonists on glutamate-induced activation in the nucleus accumbens are also absent, even though D2 receptors are functional in the D1-receptor-deficient mice, as evidenced by the preserved induction of cataplexy and expression of striatal *Fos* and *Jun* by D2-receptor antagonists.

The effects of overexpressing the gene for the D1 receptor have been investigated in two lines of transgenic mice³⁴. Increased D1-receptor binding was most marked in cortical areas. A full D1-receptor agonist caused a striking suppression of locomotion, in contrast to the dose-dependent increase in locomotor activity seen in wild-type mice. D1-receptor agonist-induced rearing and climbing behaviors were suppressed, but the transgenic animals performed as well as control mice on rotarod testing, indicating that sensorimotor coordination was unaffected. These results show that altering the levels of D1 receptor can reverse the effects of D1-receptor agonism on locomotor initiation and rearing. This raises the possibility that D1 receptors in different locations might contribute to both suppression and stimulation of movement initiation.

Two lines of mice with genetically lesioned D2 receptors have been developed^{35,43}. Impaired locomotor function has been observed in each, although there was some disparity in the degree of deficit. D2-receptor-lesioned mice also showed an abnormal pattern of long-term depression, with synaptic depression being replaced by potentiation¹³. These findings implicate the loss of D2-receptor activation in the motor system observed in PD. Antisense approaches have also been used to study D2-receptor function *in vivo*. Bilateral injection into the striata of mice of an expression plasmid that contains the antisense D2-receptor sequence led to cataplexy and inhibition of stereotypy induced by D2-receptor agonist⁴⁴. Depletion of postsynaptic D2 receptors in the rat striatum with antisense oligonucleotides diminished stereotypic sniffing in response to a high dose of apomorphine. Vacuous chewing, which is induced by low-dose apomorphine, was unaffected, suggesting the involvement of other receptors or presynaptic D2 receptors in this response. Unilateral depletion of presynaptic D2 receptors, using antisense oligonucleotides injected into rat substantia nigra, induced contralateral rotation in response to cocaine. These results suggest that presynaptic D2 receptors contribute to the locomotor responses observed with cocaine.

Mice that lack D3 receptors show increased locomotor activity and rearing behavior^{45,46}. A similar increase in spontaneous locomotor activity was observed in rats depleted of D3 receptors by injection of antisense oligonucleotides⁴⁷. Performance of the D3-receptor null mice in open-field and elevated-plus maze are characteristic of a reduced level of anxiety. The D3 receptor might also modify the response to activation of other DA receptors. These mice also show an increased sensitivity to cocaine in stimulated locomotor activity and to amphetamine in the conditioned-cue preference test⁴⁶. Locomotor stimulation induced by co-administration of D1- and D2-receptor agonists is augmented in D3-receptor null mice, but there are no differences in electrophysiological responses. Genetic lesioning of both D2 and D3 receptors leads to a more severe motor phenotype than does D2-receptor lesion



Fig. 2. Dopamine in the binding-site crevice of the dopamine D2 receptor viewed from the extracellular side. Dopamine (green) is shown binding between helices 3, 5 and 6. Sites of interaction are shown in yellow. Adapted from Ref. 28, courtesy of J.A. Ballesteros and J.A. Javitch.

alone⁴⁷. D3-receptor null mice have comparable receptor activity to wild-type mice, indicating that the D3 receptor does not contribute to autoreceptor function. Intraventricular injections of antisense oligonucleotides in rats leads to reduced D3-receptor binding, reduced neurotensin and dynorphin mRNA levels, and decreased c-fos in the cortex and Fos levels⁴⁹. These results suggest that D3 receptors may modulate neurotensin and dynorphin gene expression.

The locomotor effects of the D4 receptor appear to involve modulation of the response to activation of other DA receptors. D4-receptor null mice show increased sensitivity to cocaine, amphetamine and ethanol in locomotor tests⁵⁰. The D4 receptor might also contribute to autoreceptor function, as D4-receptor deficient mice have increased DA synthesis. A D5-receptor null mouse line has been developed but not yet fully characterized³. Injection of a D5-receptor antisense oligonucleotide potentiates the rotatory response to SKF38393 in rats with unilateral lesions induced by 6-hydroxydopamine. This contrasts with D1-receptor antisense oligonucleotides, which prevent this response. These results suggest that D5-receptor activation inhibits locomotor behavior.

Other components of the dopaminergic system have also been targeted for genetic study. Inactivating the gene for tyrosine hydroxylase produces dopamine-depleted mice that are profoundly hypoactive. Mice deficient in the gene for the DA transporter show marked hyperactivity and insensitivity to the locomotor effects of both amphetamine and cocaine. Interestingly, these mice still self-administer cocaine and the rewarding effects of morphine are augmented.

The genetic approaches to the study of the function of the dopaminergic system reveal a complex role for the various DA receptors in the modulation of locomotion. All subtypes studied by gene lesion or overexpression influence locomotor activity, either directly or via their modulation of other DA-receptor systems. In evaluating these models, it has become clear that current drugs might not differentiate between the various molecular DA-receptor subtypes *in vivo*. A recent study underscores this problem. Agonists and antagonists that were believed to be D3-receptor selective were found to have identical effects in wild-type and D3-receptor null mice⁵¹. More-specific receptor agonists are required.

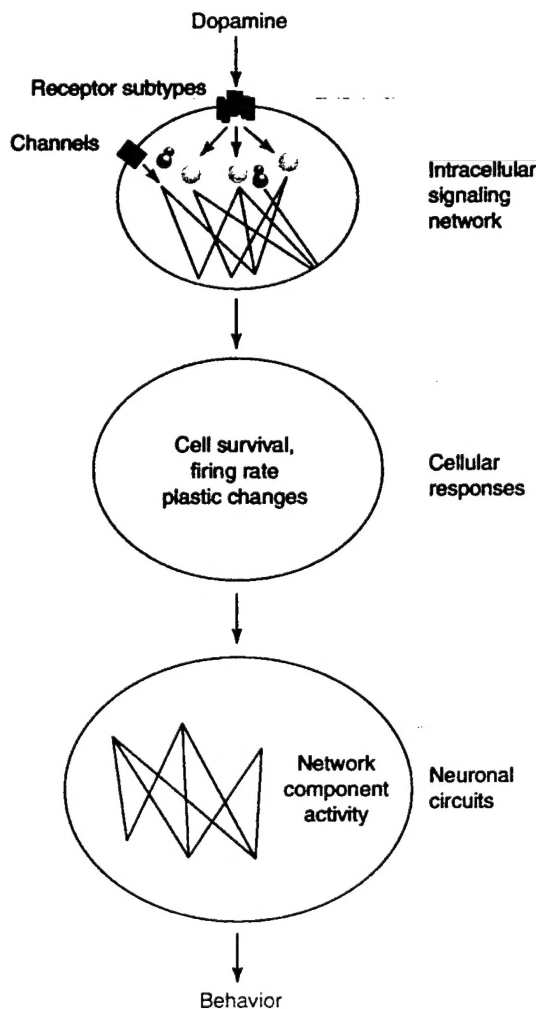


Fig. 3. Emergent properties of self-organizing network systems. The physiological response that emerges at each higher order of the system results from overall network activity and might not be fully explained by the activity of any specific individual components.

Possible role of dopamine receptors in dyskinesia and neuroprotection in PD

Dyskinesias complicate L-dopa treatment in the majority of individuals with PD. Recent studies suggest that dyskinesias result from abnormal pulsatile stimulation of DA receptors, which leads to dysregulation of downstream genes and proteins, and a consequent alteration in the firing pattern of basal-ganglia output neurons⁵². However, the precise molecular mechanism by which pulsatile stimulation of striatal dopamine receptors is translated into altered firing patterns in pallidal neurons has not yet been established. Some molecular changes associated with DA-receptor stimulation and denervation, as well as with the development of dyskinesia, are only now beginning to be revealed. DA-receptor stimulation is associated with upregulation of the expression of immediate-early genes⁵³. These gene changes can influence many aspects of neuronal function including regulation of neurotransmitters and ion channels, and thus might be crucial for long-term adaptive responses. The pattern of change in genes and G proteins is markedly altered by dopaminergic-neuron

denervation. In the 6-hydroxydopamine-lesioned rat, there are increased levels of the α subunits of G_i , G_{o1} and G_{o2} in the striatum^{54,55}. Dopaminergic-neuron denervation is also associated with increased levels of preproenkephalin (PPE) mRNA, and decreased levels of preprotachykinin in D2- and D1-receptor-bearing striatal neurons, respectively^{56,57}. These changes tend to be reversed with therapy that involves DA-like agents. However, increased PPE synthesis is not reversed by L-dopa or short-acting DA-receptor agonists that are associated with dyskinesia, but is reversed by long-acting DA-receptor agonists that do not induce dyskinesia. Indeed, several studies have demonstrated that persistent upregulation of PPE synthesis correlates with the development of dyskinesia in MPTP monkey models^{58,60}. These findings illustrate the potential of persistent stimulation of striatal DA receptors with short-acting dopamine-like agents to induce changes in gene expression that might contribute to the induction of altered neuronal circuits and abnormal behaviors such as dyskinesia.

DA-receptor activation has also been proposed as a mechanism against neurodegeneration in PD. L-dopa and its metabolites are oxidized to yield reactive oxygen species and are neurotoxic to cultured DA neurons. However, L-dopa does not induce denervation of dopaminergic neurons in normal rodent models or in primate models, suggesting that the *in vivo* protective system may be more oxidative than in vitro. This concept has, however, led to interest in the potential of DA-receptor agonists, which do not undergo oxidative metabolism, for providing neuroprotective effects. In PD there is a variety of antioxidant mechanisms, and evidence now suggests that DA-receptor agonists might also protect dopaminergic neurons through a receptor-mediated mechanism. For example, bromocriptine protection of mesencephalic neurons from L-dopa toxicity is blocked by the D2-receptor antagonist sulpiride and by antisense oligonucleotides directed against D2 receptors⁶².

Other work has used microarrays to begin to evaluate the genes that are regulated by stimulation of the DA receptor⁶³. Stimulation of DA receptors with the DA-receptor agonist pramipexole induces increased expression of multiple genes involved in synaptic plasticity and neuroprotection. It is likely that many more genes are affected by stimulation of DA receptors, and that different patterns of gene expression will result from pulsatile versus continuous stimulation of the receptor and with the use of different DA-like agents. This pattern might influence the likelihood that dyskinetic behavior will develop or that a nerve cell will degenerate.

A complexity perspective

Many elements determine the physiological effects following activation of DA receptors in the normal and parkinsonian states. Important determinants include the molecular identity of the receptor stimulated and the specific signal-transduction pathways that are activated. A variety of G-protein, ion-channel and second-messenger systems are modulated following receptor stimulation². The specific intracellular signaling pattern that results is dependent on the initial state of the cell, interaction between receptor hetero-multimers and signaling intermediaries, and the precise pattern of receptor stimulation. Receptor stimulation can induce both immediate and long-term changes in cell physiology, and these in turn can be translated into alterations in

suggests that discrete network states can exist that are only loosely tied to any particular signaling component⁶⁵. Neuroscience is succeeding in elucidating the molecular targets of DA and DA-receptor agonists, and in mapping each receptor to specific cellular and behavioral responses. The development of genome-wide profiling techniques holds the promise of even better sampling of the consequences of receptor stimulation. The challenge is to define the complex relationships between receptor activation, cell signaling, circuit activity and physiological effects in quantitative terms.

For further discussion on this topic see Box 1.

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132.9

CALPAIN UPREGULATION IN SPINAL CORD OF MPTP-INDUCED PARKINSON DISEASE MOUSE MODEL. S. Ray, G. Wilford, W. Boonen, S. Ali, N. Banik. Dept Neurol, Dept Psych, Med Univ SC, Charleston SC 29425; Div Neurotoxicol, Natl Ctr Toxicol Res, Jefferson AR 72079

Environmental toxins and genetic defects have been implicated in the development of Parkinson's disease (PD). The toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) undergoes *in vivo* oxidation by monoamine oxidase-B (MAO-B) to 1-methyl-4-phenylpyridinium (MPP⁺) which then exerts its toxic effects on dopaminergic neurons of the substantia nigra in brain. Within the central nervous system (CNS), spinal interneuronal pathways are also likely to play a significant role in the pathophysiology of PD. The main effect of MPP⁺ is mediated by irreversible inhibition of mitochondrial complex I, releasing free radicals. MPP⁺ may also activate N-methyl-D-aspartate (NMDA) receptors, increasing cytosolic concentration of free Ca²⁺. To investigate the probable involvement of the Ca²⁺-dependent cysteine protease calpain in spinal cord degeneration, we employed the mouse model of MPTP-induced PD. Male C57BL/6N mice (17 months old) were subjected to MPTP treatments (12.5mg/kg for 0.5h; 25mg/kg for 0.25h; 50mg/kg for 0.25, 0.5, 1, 2, and 24h). RT-PCR and Western blot analysis were performed using the thoracic segment of spinal cord from normal and MPTP-induced PD mice. All schedules of MPTP treatment caused calpain overexpression at the mRNA and protein levels to various extent, compared to normal mice. Calpain activity was measured indirectly by 68kD neurofilament protein (NFP) degradation, which was increased in MPTP-induced PD mice. These results suggest that calpain may play a role in spinal cord degeneration in PD. Supported by NIH-NINDS, NMSS, and AHA Foundation grants.

132.11

EXTENSIVE DOPAMINERGIC CELL DEATH IN THE RAT SUBSTANTIA NIGRA FOLLOWING INTRASTRIATAL INFUSION OF 6-OHDA: A MODEL OF PARKINSON'S DISEASE FOR NEUROPROTECTION. Yoshitsugu Oawa*, Rosario Sanchez-Pernaute, Malgorzata Kohutnicka, Andre Phillips and Kori S. Bankiewicz. Molecular Therapeutics Section, LMNN, NINDS, NIH, Bethesda, MD 20892.

Infusion of dopaminergic (DA) toxins into the striatum produces a delayed and progressive degeneration of dopamine neurons in the ipsilateral substantia nigra (SNc). The time span between lesioning and actual neuronal death provides a therapeutic window for testing neuroprotective strategies. The aim of this study was to characterize the time course and extend of cell death in the SNc following an intrastriatal infusion of 6-OHDA in order to design an optimal model for the study of neuroprotection in Parkinson's disease. Sprague-Dawley rats (n=95) received a stereotaxic unilateral infusion of 6-OHDA (0, 5, 10, 20µg in 20µl PBS) using convection-enhanced delivery (CED) to allow extended distribution within the striatum. Morphological changes were quantified by tyrosine hydroxylase immunohistochemistry (TH-IR) and apoptotic staining. Extensive DA lesion of the striatum with relative sparing of the nucleus accumbens was observed within first week. Apoptosis in the substantia nigra was determined by *in situ* end-labeling of free 3' ends in fragmented nuclear DNA and TH-IR with Nissl counterstaining. One week after the infusion, TH-IR SNc neurons showed apoptotic changes but TH-IR was preserved. Two to four weeks after the lesion, a progressive dose-dependent decrease in TH-IR cells was found in the SNc, while TH-IR cells were spared in the ventral tegmental area (VTA). This points to a particular susceptibility of nigral neurons to the apoptotic death as FluoroGold administration using the same infusion technique (0.1µl, 20µl) labeled over 90% of TH neurons in SNc and VTA. This is a valuable model of progressive and selective loss of DA neurons in the SNc, which can be used for neuroprotection approaches to Parkinson's disease.

132.13

EXTRACELLULAR SINGLE-UNIT RECORDING FROM THE STRIATUM OF FREELY-MOVING UNILATERAL 6-OHDA LESIONED RATS. M.T. Chen, B.J. Hoffer, M. Morales, C.V. Borlongan, A.F. Hoffman, P.H. Janak. Cellular Neurobiology Branch, National Institute on Drug Abuse, NIH, Baltimore, MD 21224 and Department of Neurosurgery, Neurological Institute, Veterans General Hospital-Taipei, Taiwan and Graduate Institute of Medical Sciences, National Defense Medical Center, Taipei, Taiwan.

Loss of dopaminergic (DA) input to the striatum in animals and in man produces a number of motor and sensory abnormalities. To understand how DA loss affects striatal physiology, we lesioned the medial forebrain bundle unilaterally with 6-hydroxydopamine to remove the source of DA to neurons in the striatum. Chronic recording was then used to monitor the extracellular electrical activity of ensembles of individual neurons within the striatum during behavior in the awake rat. Two weeks after 6-OHDA lesioning, arrays of 3 stainless steel Teflon-coated microelectrodes were implanted bilaterally into the dorsal striatum. One week after surgery, neuroelectric signals of 4-16 units were simultaneously amplified, filtered, sorted, and recorded in the awake subject during daily 40-minute sessions for the next 2 consecutive weeks. An increased mean neuronal firing rate of the lesioned side, as compared to the contralateral non-lesioned side, was noted. After apomorphine injection (i.e., 0.05mg/kg), the mean firing rate of neurons of the lesioned side decreased, and that of the contralateral non-lesioned side, increased. Changes in firing pattern (including mean, median, mode interspike interval, variation coefficient) were also found. In conclusion, neurons of the DA-denervated striatum of the awake rat show a higher discharge rate, and an increased inhibitory response to DAergic receptor agonists. These results suggest that altered patterns of striatal activity after loss of DA modulation may underlie the motor and sensory abnormalities seen in Parkinson's disease. Supported by the Intramural Research Program, NIDA/NIH and YGH-Taipei.

132.10

PATTERN OF GENE INDUCTION BY DOPAMINE AGONISTS IN RAT MIDBRAIN CULTURES STUDIED BY MICROARRAY ANALYSIS. J.A. Sourdis, C. Mytilineou, C.W. Olanow and S.C. Sealton. Department of Neurology, Mount Sinai School of Medicine, New York, NY 10029.

Several dopamine (DA) agonists are approved for clinical use in the treatment of Parkinson's disease. Stimulation of DA receptors by dopamine agonist improves motor features associated with this disease. In addition, several studies have suggested that dopamine agonists, including pramipexole (PPX), may have neuroprotective activity. In order to identify the gene responses that may mediate the long term effects of these agents, we are studying the response to PPX of > 4000 known genes by microarray screening using rat mesencephalic cultures. Cultures were treated with vehicle or 1 µM PPX for 6h and 24h. Total RNA was extracted from control and treated cells, reverse transcribed, labeled and used as a probe to screen microarray membranes containing DNA validated sequences of known human genes. We selected 24 genes that were regulated by >3 fold at 6 and 24 h for further analysis. Genes that are induced by PPX include V-myc, fas binding protein dax and glutathione peroxidase. The time course and response to other agonists, and to other neuroprotective and neurotoxic agents are being investigated. These studies will help elucidate the mechanisms underlying the long term symptomatic and putative neuroprotective effects of dopamine agonists.

132.12

ALTERATIONS IN THE ABUNDANCE, COMPOSITION AND PHOSPHORYLATION OF STRIATAL NMDA RECEPTORS IN THE RAT 6-OHDA MODEL OF PARKINSON'S DISEASE.

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NMDA glutamate receptors have a potent modulatory effect on dopaminergic signaling, and are potential targets for new therapeutics in human Parkinson's disease. We used quantitative immunoblot and immunoprecipitation methods to examine alterations in the relative abundance, subunit composition and phosphorylation of NMDA receptors in the rat unilateral 6-hydroxydopamine (6-OHDA) model of Parkinson's disease. In striatal membranes, the abundance of NR1 and NR2B proteins were reduced on the lesioned side (39% and 35%, respectively) while the abundance of NR2A was unchanged. Coimmunoprecipitation of soluble striatal membrane proteins under native conditions showed a marked reduction in NMDA receptor complexes composed of NR1 and NR2B subunits. The serine phosphorylation of NR1 at ser890 and ser896, but not ser897, and the tyrosine phosphorylation of NR2B but not NR2A were decreased in the lesioned striatum. Chronic treatment with L-DOPA normalized the abundance and composition of the NMDA receptors found in striatal membranes, but also produced hyperphosphorylation of NR1, NR2A, and NR2B subunits. These data suggest that assembly and insertion of NMDA receptors into striatal membranes are modulated by dopamine, and that altered receptor subunit phosphorylation may make an important contribution to the adverse effects observed after long-term L-DOPA treatment.

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132.14

SPONTANEOUS LONG-TERM COMPENSATORY DOPAMINERGIC SPROUTING IN MPTP-TREATED MICE. S. Dawson*, E. Borod, T. Borad, B. Houlac and C.E. Gross. Basal gangl., CNRS UMR 5543, Université Bordeaux 2, 33076 Bordeaux France

Several recent reports would seem to indicate that surviving adult DA neurons in animal models of Parkinson's disease can undergo plastic trophic support-induced modifications similar to those already observed in glutamatergic and serotonergic striatal afferents. The spontaneous compensatory sprouting of DA fibers after partial nigral degeneration has already been elicited in the rat (David *et al.*, 1984; Olanow *et al.*, 1986; Blanchard *et al.*, 1996) and in the mouse (Ho and Blum, 1998) but the consequences of severe degeneration have not yet been explored.

The present study was designed to investigate whether this phenomenon can still be observed after severe MPTP intoxication in the mouse. [³H]-DA uptake was measured by striatal synaptosomal preparation in OF1 mice at 0.5, 1.5, and 7 months after intoxication.

Although the number of TH immunoreactive neurons remained stable in the SNc throughout the entire protocol, a progressive increase in [³H]-DA uptake was observed from 17% at 0.5 months uptake increased to 65% at 7 months (p<0.05).

These results provide clear evidence that spontaneous long-term compensatory dopaminergic sprouting can occur even after severe MPTP-induced nigral degeneration. The origin of the new DA fibers remains a mystery. The sprouting we report could be due either to regrowth from surviving DA nigral neurons or to the proliferation of intrinsic striatal DA neurons or to both.

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